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METHOD OF QUANTIFYING BINDING

Field of Invention

The present invention relates to a method of quantifying binding of an agent to a specific binding partner, calibration products and uses thereof. The present invention, especially but not exclusively, is for use in blot based detection techniques and relates to quantification of the amount of an agent in a sample.

Background to the Invention

Separation techniques such as blot-based techniques can be used to identify the presence of a particular target molecule in a sample. One blot-based technique, Western blotting, can be used to identify the presence of a particular protein in a sample through its interaction with an antibody specific for said protein. The proteins of a sample may be separated from each other by electrophoresis, transferred to a suitable membrane support, which is then interrogated with the specific antibody. The binding of antibody to the protein is visualised as a "spot" on the membrane, providing information as to its presence and location. The location may give information about the physical state of protein, for example glycosylation, phosphorylation or proteolysis.

The disadvantage of Western blotting is that the data generated are qualitative (unitless), which limits the information obtained from an experiment and does not provide quantitative results comprising units. Furthermore, considerable day-to-day variation in sensitivity is observed, which prevents ready comparison between experiments performed on separate occasions, particularly when the experiments are performed by different researchers. Accordingly, there is considerable inter and intra experimental variation which makes comparisons between experiments difficult and inaccurate. These shortcomings limit both the quality of information gained and the productivity of the technique.

There are a variety of other blot based detection techniques. Southern blotting is a technique used to detect the presence of a particular DNA sequence, whilst Northern blotting is used to locate a particular RNA sequence within a mixture. ELISA techniques are highly sensitive, and therefore able to detect very small amounts of protein or other antigenic substance in a sample. The basis of this method is the binding of the antigen by an antibody that is linked to a surface of a plate. Formation of an immune complex is detected by use of peroxidase coupled to an antibody, the peroxidase being used to generate an amplifying colour reaction. However, despite the highly sensitive nature of ELISA, it does not quantify the amount of protein or antigen present in the sample. Thus, the disadvantages described in connection with Western blotting are also relevant to other blot based detection techniques.

There are other separation based techniques such as High Performance Liquid Chromatography (HPLC) and isoelectric focusing. Isoelectric focusing techniques are techniques used to separate proteins which utilise differences in the isoelectric points of the proteins. The isoelectric point of a protein is the pH at which a protein has no net charge. Under those circumstances it will not migrate in an electric field. Isoelectric focusing techniques use a pH gradient set up between a cathode and an anode and proteins will migrate towards their isoelectric point. Isoelectric focusing techniques do not provide truly quantitative results.

It is therefore long been desired that simple, effectively reproducible calibration technology would correct the described shortcomings and provide quantitative data which are readily comparable between experiments.

Statements of Invention

In the broadest aspect, the present invention provides a method of quantifying a target moiety in a sample which may contain the target moiety, the method comprising using a specified concentration or varying the concentration of a presentation system in order to generate a comparison point or calibration curve which provides means for comparing a signal generated by the presentation system and a signal generated by

a sample, wherein said presentation system comprises at least one copy of said target moiety or part thereof.

In a first aspect of the invention, there is provided a method of quantifying the amount of target moiety in a sample which may contain the target moiety, the method comprising:

a) providing a presentation system which comprises at least one copy of the target moiety or part thereof that is recognisable by a binding partner and at least one domain which is non-reactive to said binding partner,

b) carrying out a separation detection technique on said presentation system, wherein said presentation system is present in a specific concentration;

c) generating at least one comparison point comprising intensity of a signal produced by the presentation system versus the concentration of the presentation system.

Preferably, the method comprises a further step of comparing the comparison point or multiple comparison points with the sample to quantify the amount of target moiety present in the sample. Preferably, the presentation system is of a known molecular weight. It is of particular advantage to have knowledge of the molecular weight of the presentation system in order to compare the presentation system with the sample which may contain the target moiety or part thereof. In one embodiment, the presentation system is present in a single concentration. In this embodiment, a single comparative point will be generated. Thus, in this embodiment, the presentation system could be used as an internal standard. This could be used to confirm that detection of a target molecule has been performed correctly, wherein the presentation system operates as a positive control and a reference signal. This is particularly important in circumstances where the target molecule is absent from a sample (see example of Figure 4).

It is envisaged that embodiments of the present invention may be used in the comparison of the protein repertoire of part or all of a cell or tissue is made between

samples derived from different physiological states (e.g. states of development, disease vs. control etc.). It is now possible to stain for certain forms of post-translational modification of proteins, such as phosphoproteins and glycoproteins. The incorporation of an internal standard comprising a presentation system comprising a target moiety which is a suitably modified peptide structure (phosphopeptide, glycopeptide or other modified peptide) into the experimental design would improve quantitation of data and would permit the ready comparison of results between experiments run in parallel or on separate occasions,

In an alternative embodiment, the presentation system is present in a series of varying concentrations. In this embodiment, multiple comparison points may be generated and thus a calibration curve may be produced.

Throughout the specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Reference herein to presentation system is intended to include, but not be limited to a molecule which comprises one or more linear units or domains which are linked in tandem. In one embodiment, the presentation system comprises one or more identical units or domains. In an alternative embodiment, the presentation system comprises differing units or domains. The presentation system may also be referred to as a calibration standard. The presentation system also comprises at least one copy of the target moiety or part thereof. In a preferred embodiment, the presentation system is of known molecular weight. In one embodiment, the presentation system may comprise a concatamer. In an alternative embodiment the presentation system may be a nucleic acid molecule. Alternatively, the presentation system may be a peptide or protein. In a particular embodiment, the presentation system may be a naturally produced protein or domain. In an alternative embodiment, the presentation system may be an artificial protein or domain.

The presentation system may comprise a mixture of different types of units or domains. The units or domains may be of known molecular weight and be blind to the binding partner specific to the target moiety or part thereof i.e. non-reactive thus, the units or domains of the presentation system may be considered "immunologically blind" or "reactively inert".

In one embodiment, the comparison point or calibration curve generated may then be used to quantify the amount of the target moiety in a sample. In particular, the comparison point or calibration curve generated by the present invention plots the intensity of the signal produced by the target moiety or part thereof as part of the presentation system against the concentration of the presentation system. A further separation based detection technique may then be carried out on a sample which may contain the target moiety. Alternatively, a further separation based technique may be carried out on a sample at the same time as the presentation system. The intensity of a signal, if any signal is observed, may then be determined. The comparison point may then be used to express the amount of target moiety in the sample relative to the amount of target in the calibration point, or calibration curve to determine the concentration of the target moiety and thus the amount of target moiety present in the sample. The signal produced by the sample or presentation system may be, for example and without limitation, an enhanced chemiluminescent substrate. Other methods of detection include chromogenic substrates for enzyme catalysed detection, which are insoluble colour products, such as 4-methoxy-naphthol/H_2O_2 for peroxidase based anti-IgG, or fluorescence based detection systems which utilise fluorophores attached to a detecting reagent such as antibody or protein A or protein G. Radioactivity ^{125}I labelled antibody or protein A or protein G. In an alternative embodiment, methods of detection involving the binding of dyes to the presentation system could be utilised. Dyes that could be used include, but are not limited to Pro-Q diamond (Molecular Probes, OR, USA), which is a dye for protein containing phosphorylated amino acids and Pro-Q emerald (Molecular Probes, OR, USA) which is a dye for carbohydrate containing moieties. Other binding partners could be other chemical molecules, such as a drug or candidate drug molecule.

In one embodiment, when the presentation system is a protein or peptide, it may comprise a number of stably folded protein domains. The number of domains may be varied in order to vary the molecular weight of the presentation system. In one preferred embodiment, at least one domain contains an amino acid capable of accepting a covalent bond from the target moiety or part thereof. The domains of the presentation system, apart from the target moiety or part thereof, are inert, that is to say, these domains are non-reactive to the specific binding partner of the target moiety or part thereof. The "inert" domains control the molecular weight of the presentation system to facilitate multiplexing of samples. Reference herein to multiplexing means the ready deconvolution of information derived from a procedure using a mixture of test and presentation system sample to obtain the signal derived from the test component and the signal derived from the presentation system.

Thus, in one embodiment, the presentation system is present in one channel of a blot and a sample which may comprise the target moiety is present in a separate channel. In such an embodiment of the present invention the method allows simultaneous examination of the presentation system and a sample which may contain a target moiety in a single experiment. This embodiment advantageously provides an unambiguous distinction between the presentation system and the sample, despite the separation based technique being carried out on both at the same time.

Alternatively, the presentation system and the sample may be present in the same channel or lane of the blot.

What form the presentation system takes is dependent on the form of the target moiety to be detected and quantified. If, for example, the target moiety to be quantified is a nucleic acid, then the presentation system may comprise a sequence of nucleic acids. The presentation system may comprise a sequence of DNA units or domains of known molecular weight. Alternatively, the presentation system may comprise RNA units. Alternatively, if the target moiety is a peptide epitope or protein, then the presentation system will also comprise peptide units or domains,

and Western blotting or ELISA can be used to quantify the amount of the target moiety present in the sample. It is also envisaged that heterocombinations may make up the presentation system i.e. the presentation system may comprise both peptide and nucleic acid.

In a particular embodiment, the presentation system may comprise one or more domains, such as I27, from titin. Titin contains a number of β-sandwich domains belonging to the Ig family. The I27 domains usually contain two cysteine residues and fold to form stable structures of 10kDa. In Nature, the I27 domain contains two cysteine residues (the site for covalent attachment of peptide), however mutation of these cysteine residues, to serine for example, is compatible with domain folding. Thus a presentation system of I27 can be formed where the molecular weight step size is a convenient unit (10kDa steps) and where one unit (or more if required) can be engineered to possess a single cysteine residue for peptide attachment while all other units of I27 will lack cysteine residues. In alternative embodiments, the units of I27 may lack other reactive residues. These residues may include, but not be limited to lysine, glutamate and aspartate. A copy of I27 could contain one or more of these reactive residues, offering a controlled number of sites for the covalent attachment of target moieties.

The titin domains used in the presentation system may be wild-type domains. Alternatively, one or more of the titin domains may be mutated to possess either one or no cysteine residues. In one embodiment, the presentation system comprises one or more I27 domains and a copy of a target moiety, wherein one of the I27 domains comprises a single cysteine residue and the other I27 domains lack a cysteine residue. In a preferred embodiment, the presentation system comprises five I27 domains.

Reference herein to target moiety or part thereof includes, but is not limited to sequences of DNA, RNA or peptide. The target moiety may further include saccharides, metabolite cofactors, haptens or modification groups. Modification

groups may include phosphate, nitrosylated groups, sulphated groups or glycosylphosphatidyl inositol (GPI) groups.

The target moiety or part thereof may be a peptide or protein sequence. Alternatively, or in addition, the target moiety or part thereof may be an epitope or antigenic sequence. The position of the target moiety or part thereof within the linear sequence of the presentation system may vary. In one embodiment of the present invention, the presentation system may have one copy of the target moiety or part thereof present in its sequence. In an alternative embodiment, the presentation system may comprise more than one copy of the target moiety or part thereof. In a particular embodiment, the presentation system may comprise differing target moieties or parts thereof. The target moiety or part thereof may be continuous within the presentation system. Alternatively, the target moiety or part thereof may be discontinuous within the presentation system i.e. only in its binding state when the presentation system is in its folded state, thus presenting the target moiety or part thereof such that the specific binding partner can only bind to the target moiety or part thereof when the presentation system is in its 3-D form. Reference herein to protein or product is intended to include: protein complexes or fragments; enzymes; enzymatic products or conjugates; primary metabolites; hormones or antibodies.

When the presentation system and/ or the target moiety is a protein or peptide, the isoelectric point may be controllable. This particular embodiment provides particular advantages when the separation technique used is 2-D electrophoresis.

In a particular embodiment of the present invention, the target moiety comprises the protein SERCA2a. The presentation system may comprise an epitope of SERCA2a. SERCA2a is the cardiac muscle isoform of the sarcoplasmic reticulum Ca2+-ATPase family. In a preferred embodiment, the epitope of SERCA2a comprises the amino acid sequence LEPAILE.

In an alternative embodiment, the target moiety is the protein SERCA2a which has been phosphorylated on serine-38. Preferably, the presentation system comprises an epitope from this protein. Preferably, the epitope comprises the amino acid sequence ^{31}KLKERWGS(PO_4)NEL^{41}

The separation technique, for example a blot based technique, may be carried out on the presentation system such that the presentation system migrates as a well focused or distinct band, which is separate from SERCA2a in the sample. The present method enables the user to unambiguously derive the presentation system from the sample, once the separation technique has been carried out. Thus, the present invention provides a method which enables the generation of truly quantitative data from separation technique experimentation.

Reference herein to specific binding partner refers to any molecule which has a specific binding affinity for the target moiety and is capable of binding thereto. The binding partner may be detectable using blot based detection techniques. The binding partner may be the same species as the target moiety e.g. polypeptide to peptide binding or nucleic acid polymer to nucleic acid polymer. Alternatively, the specific binding partner may be a different species to the target moiety for example nucleic acid polymer binding to peptide/polypeptide; dye binding to peptide/polypeptide. In one embodiment, the binding partner is an antibody. Antibodies raised to the target molecule may be monoclonal antibodies or polyclonal antibodies. If the binding partner is an antibody, it may specifically bind to a target moiety which comprises a peptide epitope. In an alternative embodiment, the binding partner might be another protein naturally occurring (e.g. far western blot) or unnatural (e.g. fluorobody). In an alternative embodiment, the binding partner may comprise a DNA sequence. In a yet further alternative embodiment the binding partner may comprise a RNA sequence. Alternatively, the binding partner may be a drug. The presentation system may be used to review the potential targets of a candidate drug molecule. The separation technique may be 2D electrophoresis. Alternatively, other separation techniques may be used. A presentation system comprising a target molecule or part thereof which is

capable of binding to a drug molecule, which is the specific binding partner. The intensity of a signal produced by the presentation system may then be compared with the signal intensity of the sample.

In an alternative embodiment, the binding partner may be a stain or dye. One such stain is Pro-Q Emerald (Molecular Probes), which may be a binding partner when the target moiety or part thereof is a glycoprotein. A further example of a stain as a binding partner is Pro-Q Diamond (Molecular Probes), which may bind to a target moiety comprising a phosphoprotein. When the binding partner is a dye or stain, the domain(s) of the presentation system must be "silent" and must not be recognised or detected by the dye or stain.

Preferably, the binding partner is an aptamer. Aptamers are novel synthetic DNA or RNA ligands, which have been defined as artificial nucleic acid ligands that can be generated against amino acids, drugs, proteins and other molecules. Aptamers may be double-stranded DNA ligands or single stranded RNA ligands. They are isolated from complex libraries of synthetic nucleic acids by an iterative process of adsorption, recovery and reamplification (SELEX). RNA aptamers are nucleic acid molecules with affinities for specific target molecules. They have been likened to nucleic acid antibodies because of their ligand binding properties.

The presentation system may further comprise a tag or detectable molecule. The tag may carry out the function of purification of the presentation system. In one embodiment, when the presentation system comprises a peptide or protein, the tag may be a peptide tag such as a tag which may possess enzymatic activity to convert a substrate to a form that is readily detectable by an assay. It will be appreciated that the position of the tag may be at the amino terminal or carboxy terminal or inserted internally with respect to the amino acid sequence of the presentation system. Other examples of tags or detectable molecules may include, but not be limited to, a His-tag or a fluorescent label such as GFP. In an alternative embodiment, the tag may comprise the target moiety or part thereof of the presentation system.

Reference herein to separation based detection experiments or techniques can be taken to include, but not be limited to, blot based detection techniques such as immunological assays based on detection by an antibody or other type of specific binding partner specifically recognising the target moiety, either when present in the presentation system or in a sample. Such assays include dot blots, Western blot, RIA and fluorescence polarisation. Alternatively, the method of the present invention may utilise other blot based detections experiments or techniques such as Northern blotting or Southern blotting or PCR. Separation based techniques may also include High Performance Liquid Chromatography (HPLC), capilliary electrophoresis, mass spectrometry and isoelectric focusing and combinations of the above (e.g. 2D electrophoresis, IEF/SDS-PAGE). It is envisaged that the method of the present invention may also be used in techniques such as ELISA.

The present invention delivers controlled, predictable manufacture of presentation systems. Furthermore, it will deliver calibration standards displaying the high quality characteristics including near homogeneous electrophoretic behaviour of presentation systems and multiplexing of test samples and presentation system samples. This has the advantage of increasing sample throughput.

In a second aspect of the invention, there is provided a product (presentation system) for use in quantifying the amount of a target moiety which may be present in a sample, the product comprising a presentation system, said presentation system comprising at least one copy of a target moiety or part thereof and further comprising at least one domain linked to said target moiety, wherein the domain(s) is/ are of known molecular weight and are non-reactive to a binding partner specific to said target moiety or part thereof.

Preferably, the product further included any one or more of the features hereinbefore described. Thus, the present invention further provides calibration standards which are simple and reliable and user-friendly and also enables the results of experiments carried out on separate occasions to be compared accurately.

In a third aspect of the invention, there is provided a kit for quantifying the amount of a target moiety in a sample, the kit comprising a presentation system, said presentation system as hereinbefore described. Optionally, the kit further comprises a binding partner specific to the target moiety or part thereof. The domain(s) of the presentation system may be "inert" or blind to the binding partner, i.e. do not bind to the binding partner. Optionally, the kit may comprise instructions for use thereof.

In one embodiment of the invention, the presentation system is provided in a kit as a positive control sample. The kit further comprises an antibody product. The antibody product may be the binding partner. The presentation system comprises a target moiety or part thereof which will react with the antibody binding partner and thus provide a positive control.

In a fourth aspect of the invention there is provided the use of a product (presentation system) as hereinbefore described for quantifying the amount of a target moiety which may be present in a sample.

In a fifth aspect of the invention there is provided a method for quantifying the amount of SERCA2a protein in a sample, the method comprising:

a) providing a protein which comprises at least one copy of an epitope of SERCA2a that is recognisable by an antibody and at least one I27 domain from titin protein which is non-reactive to said antibody, wherein said protein is of a known molecular weight;

b) carrying out a Western blot on said protein, wherein said protein is in a specific concentration;

c) generating at least one comparison point comprising intensity of a signal produced by the protein versus the concentration of the protein.

Preferably, the protein is in a series of varying concentrations and the comparison point comprises a plurality of comparison points, thus providing a calibration curve.

Preferably, the comparison point or calibration curve is then used to determine the amount of SERCA2a protein present in a sample by comparison of the intensity of signal produced by the sample with the calibration curve. In a preferred embodiment, the epitope comprises the amino acid sequence YLEPAILE.

In an alternative embodiment, the SERCA2a protein is phosphorylated on serine-38. Preferably, the protein comprises an epitope of the phosphorylated SERCA2a protein. In a particular embodiment, the epitope comprises the amino acid sequence ^{31}KLKERWGS(PO_4)NEL.^{41}

In a sixth aspect of the invention, there is provided a method for quantifying an amount of a peptide epitope in a sample, said method comprising:

(a) providing a protein presentation system comprising at least one copy of the peptide epitope and at least one further peptide domain, wherein said presentation system is of known molecular weight;

b) carrying out a Western blot experiment on said presentation system, wherein said presentation system is in a specific concentration; wherein said Western blot experiment utilises a binding partner specific to the target moiety; and further wherein said peptide domain of the presentation system is non-reactive to the binding partner; and

c) generating a comparison point comprising intensity of a signal produced by the presentation system in said technique versus the concentration of the presentation system.

Preferably, the presentation system is in a series of varying concentrations and said comparison point is a plurality of comparison points which may be used to produce a calibration curve. In one embodiment, control of the number of target moieties within the presentation system can be achieved by selecting a protein with the desired number of reactive residues or by engineering the sequence of the protein such that it contains a limited number of acceptor sites for covalent attachment of the peptide epitope. In a preferred embodiment there is only one site for covalent attachment. In

alternative embodiments there may be more than one site for the peptide epitope to attach to a protein domain to form a presentation system. An advantage of the present invention is that the molecular weight of the presentation system is controllable. This is achieved by employing a presentation system made up of a number of stably folded domains. In this embodiment, the domains are protein domains. By varying the number of domains in the presentation system, the molecular weight of the presentation system can be varied. In a preferred embodiment, one of these domains contains an amino acid capable of accepting a covalent bond from a modified epitope peptide. The other domains are inert in this sense, but are present to control the molecular weight of the presentation system to facilitate multiplexing of samples and to facilitate the separation of multiple presentation system components, where multiple components exist (e.g. single shot applications, internal standards for 2D electrophoresis).

The target moiety may be incorporated into the presentation system using a variety of known methods. In a particular embodiment, the target moiety may be conjugated to the presentation system. Alternatively, if the target moiety is a protein or peptide and the presentation system is also a protein or peptide, a DNA segment encoding the target moiety may be incorporated into the DNA encoding the presentation system, and subsequently expressed with the presentation system using known methods of protein expression.

The present invention advantageously enables accurate control of the molecular weight of the presentation system, across a wide Mr range (10kDa –250kDa or greater, or smaller if required). As a result, the present invention will deliver flexibility in the multiplexing of standards and test samples. It will also permit the development of an advanced format of the calibration standard technology, which allows the dispensing of the calibration standard range in a "single shot" thus maximising convenience for the end-user. In a preferred embodiment, a series of presentation systems, which may have differing molecular weights, are subject to a separation technique whilst loaded in a single channel. This embodiment provides the

user with a variety of calibration standards to compare with samples if required. In a preferred embodiment, the method of the present invention utilises a plurality of presentation systems which may be used in a single experiment to provide a range. The plurality of presentation systems may be of differing molecular weight and may be mixed together in specified molar ratios to achieve a range of presentation systems which may be used in a single separation based experiment.

Detailed Description

The invention will now be described by way of example only, with reference to the accompanying figures, in which:

Figure 1 shows a presentation system according to one embodiment of the present invention. Figure 1 shows the Atomic structure of I27 domain of titin. Location of cysteine C47 (site for covalent attachment of epitope peptide) is highlighted

Figure 2 shows a schematic representation of a presentation system comprising mutant forms of I27. A synthetic gene encoding five copies of I27 in series is displayed. Copies 1,2,4 & 5 of I27 lack cysteine residues, and copy 3 retains a single cys for peptide attachment. (His)6 module is for product purification. Linker regions contain unique sequence & restriction sites.

Figure 3: shows the specific recognition of a serine-38 presentation system (calibration standard). A) Schematic representation of the calibration standard (referred to as calibration-38). Epitope peptide was covalently bonded to a cysteine residue in the third domain of an $(I27)_5$ concatamer as described in "Experimental Procedures". B) Calibration-38 composition described by electrospray mass spectroscopy. The product (calibration-38) is separated from substrate $(I27)_5$ components and a contaminant. Components marked with * were included in the calculation of product yield (5.4%). C) Calibration-38 (0.06-3.2pmol) and calibration-aCLEP (0.3-60pmol total (I27)5 protein) was analysed by Western

blotting with antibody SERCA PS-38 (1:5000 dilution) following electrophoresis in 10% SDS-PAGE gels. SERCA PS-38 detected the calibration-38 product (~60kDa) at loadings of 0.1pmol and above.

Figure 3C shows that only calibration-38 is recognised by anti-SERCA-38 antibody, which confirms that the presentation system is immunologically silent.

Figure 4 shows SERCA phosphorylation in rat cardiac myocytes is not detected by antibody SERCA PS-38. Thus, Figure 4 is a negative result. Isolated rat ventricular myocytes were paced electrically at 0.5Hz for 5 min unless otherwise stated. Cells (A; 10,000; B; 2,500; rod shaped cell count) were treated without further intervention (control lane 1) or with 1 μM isoproterenol (lane 2); exposured to increased stimulation frequency (2.5 Hz; lane 3); exposed to 2.5 mM extracellular calcium (lane 4); or exposed to 1 μM calyculin A (lane 5). A) Calibration-38 (concentration range 0.04 to 4 pmol) and rat myocytes were submitted to SDS-PAGE (10 % polyacrylamide) and transferred to PVDF membrane. Blot was probed with antibody SERCA PS-38 (1:5000) and visualized with goat anti-rabbit IgG peroxidase together with an enhanced chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) B) In a parallel experiment, myocytes were separated by 15 % SDS-PAGE and transferred to PVDF membrane. Threonine 17 phosphorylated phospholamban was detected using antibody PT-17 (1:5000).

Figure 5: shows a SDS-PAGE gel carrying varying concentrations of a presentation system for SERCA2a protein in different lanes and a single lane containing a sample comprising SERCA2a protein. Figure 5 shows an embodiment of the present invention being used as a positive control.

Examples

Example 1

The method of the present invention was used to detect the protein SERCA2a in a sample. A presentation system (calibration standard) for antibody α-CLEPAILE, which recognises the C-terminus of SERCA2a, was constructed as described for calibration SERCA-PS38 by reacting 0.1micromole peptide YLEPAILE (single letter

codes) with 5micromole sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC); purification of peptide-cross-link complex by gel filtration chromatography; and incubation of the peptide-cross-link product with $(I27)_5$ (0.02micromoles) in the presence of 9M urea. Product was dialysed against water and the protein concentration determined using a BCA assay.

Quantitation of SERCA in a sample of cardiac muscle sarcoplasmic reticulum. Cardiac sarcoplasmic reticulum (10μg) and calibration-CLEPAILE standards (15 – 0.05 pmol) were separated in individual lanes on a 10% SDS-PAGE gel. The samples were transferred to PVDF membrane and probed with an antibody specific for the SERCA2a sequence LEPAILE (1:5000 dilution). Antibody binding to its epitope was detected using goat anti-rabbit IgG-peroxidase and a commercial chemiluminescent substrate preparation (Pierce). Chemiluminescence was detected using a CCD camera (Figure 5).

Quantitation of the amount of SERCA2a in the sample can be achieved by analysis the band intensity of samples and calibration standards by densitometry. A plot of optical density (corrected for background signal) against quantity of the presentation system (calibration standard) should be prepared. This plot is considered to be a calibration standard curve. The SERCA2a protein content of the sample can be calculated using the calibration standard curve, by converting the optical density signal (corrected for background) of that sample to pmoles of epitope from this plot.

The above Example shows a way in which the presentation system and the method of the present invention may be used to positively identify a target moiety in a sample and to quantify the amount of target moiety in a sample.

Example 2

Example 2 shows a way in which the presentation system and the present method may be used to negatively identify a target moiety in a sample i.e. wherein the target moiety is not present in the sample.

The method of the present invention was used to detect phosphorylation of sacroplasmic reticulum Ca2+-ATPase (SERCA2a) on serine-38. A standard Western blot approach had failed to detect serine-38 phosphorylated Ca2+-ATPase in either kinase treated sarcoplasmic reticulum vesicles and isolated rat ventricular myocytes.

The phosphorylation of the cardiac muscle isoform of the sarcoplasmic reticulum Ca2+-ATPase (SERCA2a) on serine-38 has been described as a regulatory event capable of very significant enhancement of enzyme activity. Independent confirmation of these observations has not been forthcoming. A polyclonal antibody, wholly specific for the phosphorylated serine-38 epitope on the Ca2+-ATPase, was utilised to evaluate the phosphorylation of SERCA2a in isolated sarcoplasmic reticulum vesicles and isolated rat ventricular myocytes. A quantitative Western blot approach failed to detect serine-38 phosphorylated Ca2+-ATPase in either kinase treated sarcoplasmic reticulum vesicles, or suitably stimulated cardiac myocytes. The presentation system of the present invention confirmed that the detection sensitivity of assays (0.03-0.1pmol) was adequate to detect phosphorylation of just 1% of Ca2+-ATPase molecules on serine-38. Although a phosphoprotein of 100kDa was evident in rabbit cardiac SR preparations, it was not recognised by the phospho-serine-38 specific antibody (2).

Preparation of phospho-specific antibodies.

Phosphorylated Ca^{2+}-ATPase peptide on Ser-38 residue (^{31}KLKERWGS(PO4)NEL^{41}) was prepared by the CaMKII phosphorylation of peptide ^{31}KLKERWGSNEL^{41}. The phosphopeptide was purified to homogeneity by reverse phase high performance liquid chromatography. Peptide was conjugated to keyhole limpet haemocyanin (KLH) using carbodiimide cross linkage (3) and dialysed extensively against buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl). Adult New Zealand White rabbits were immunised with ~150µg KLH and attached peptide at 6 weeks intervals and immune serum collected 11 days after immunisations. Serum was prepared and stored at –70°C. A polyclonal antiserum is described herein: SERCA PS-38 raised to the phosphorylated peptide.

Production of an embodiment of the presentation system comprising a scaffolding protein comprising titin I27 domains (denoted $(I27)_5$).

A gene encoding a concatamer of mutant forms of the I27 domain of titin was used. The construct differs from the one described in Brockwell et al. (4) in that the two C-terminal cysteine residues have been deleted (Fig. 2A). It is referred to as $(I27)_5$ throughout this study. $(I27)_5$ was expressed and purified as described in Brockwell et al.. (4).

Presentation System: An embodiment comprising peptide conjugated to a concatamer

Purified phosphorylated Ser-38 peptide (^{31}KLKERWGS(PO$_4$)NEL^{41}) (0.1 μmol) was mixed with an excess of sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) cross-linker (5 μmol) in buffer containing 0.1M sodium phosphate, 0.15 M NaCl, pH 7.2. After 1 h of incubation at room temperature, the maleimide-activated peptide was purified by gel filtration chromatography using a Superdex Peptide HR 10/30 column (Pharmacia Biotech). The chromatography was performing using 0.1M sodium phosphate, 0.15 M NaCl, pH 7.2 and a flow rate of 0.25 ml/min. Fractions of interest were pooled and urea was added to the fractions to make a final urea concentration of 9M. $(I27)_5$ concatamer (0.1 μmol) was added to the mixture and incubated for 2 h at room temperature. The conjugate was dialysed extensively against water. Final product (the presentation system according to the present invention) was stored at –20°C. The same procedure was followed to conjugate SERCA2a peptide (YLEPAILE) to $(I27)_5$ concatamer to form an alternative presentation system. Protein concentration was determined by a BCA assay (5).

Immunoblot analysis.

Myocardial proteins were separated by SDS-PAGE using 10 % and 15 % polyacrylamide gels as described by Laemmli (8). Following separation, proteins were transferred to PVDF membranes (Pall BioSupport, Portsmouth, UK) by semi-dry blotting, and nonspecific binding sites were blocked for 2 - 4 h at room

temperature using 5 % dried milk and Tris-buffered saline (pH 7.4), 0.1 % Tween 20. Membranes were probed overnight at 4°C with primary antibodies: PT-17 (1:5000) for the Thr-17 phosphorylated form of phospholamban (6); α-CLEP (1:5000) for SERCA2a (16); and SERCA PS-38 (1:5000) antiserum specific for the Ser-38 phosphorylated form of Ca^{2+}-ATPase. A secondary horseradish peroxidase-labeled antibody raised in rabbit (Goat Anti-Rabbit IgG (H+L); Jackson Immunochemicals; lot number 38179) or protein A peroxidase (Sigma) were used in combination with two enhanced chemiluminescent detection system (SuperSignal West Pico Chemiluminescent Substrate and SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) to visualize the primary antibodies. Data were captured using a Fuji LAS-1000 Imaging System CCD Camera (AIDA software for analysis).

Results: The phosphorylation of SERCA2 on Ser-38 has been described as a regulatory feature capable of very significant activation of Ca2+-ATPase activity. This site, although unique to SERCA2, is contained within a segment of the protein which is highly conserved between SERCA1 and SERCA2 particularly from residue 39 onwards. As such, the two proteins are likely to display comparable structures in this region. By analogy with SERCA1, for which two high resolution structures exist the Ser-38 site of phosphorylation on SERCA2 is predicted to be in a surface exposed, highly mobile segment of the protein. This segment remains solvent exposed in both conformational extremes of the enzyme (E1, E2;), which would make it accessible to the kinase and phosphatase in these states. These properties also lend themselves to antibody binding to the site, as it is surface exposed, and highly mobile. We have produced a phosphorylation-site specific antibody to this feature in an effort to define the incidence and role of Ser-38 phosphorylation in cardiac muscle. A polyclonal antibody was produced to the sequence ^{31}KLKERWGS(PO4)NEL^{41}, phosphorylated, as shown, at Ser-38 This polyclonal antiserum, SERCA PS-38, was wholly specific for the phosphorylated peptide, as the phosphopeptide was a potent inhibitor of antibody binding to antigen (IC50 18nM), whereas the equivalent dephosphorylated peptide was unable to interfere with antibody:antigen recognition.

SERCA PS-38 recognition of a calibration standard (presentation system): Having confirmed that polyclonal antibody SERCA PS-38 was wholly specific for the phosphorylated Ser-38 epitope we examined the phosphorylation status of this residue in SERCA following exposure of cardiac SR vesicles to CaMKII. SERCA was not detected in these Western blot experiments. It was important to establish the basis of this negative result, to ensure that it was providing information about the incidence of Ser-38 phosphorylation, rather than recording a technical failing of the antibody or experiment. To this end, we constructed a presentation system comprising a target moiety, in this case, the phosphopeptide epitope, attached to an inert scaffold protein of known molecular weight. The scaffolding protein was chosen as it contained a single site for peptide attachment (Fig 3A) thus providing a uniform structure for the presentation of epitope peptide, ideal for accurate quantitation.

The presentation system employed comprised a concatamer of five copies of a domain from titin (I27), mutated to remove all but one cysteine residue from the concatamer sequence (Figure 3A). The target moiety, in this case purified phosphoepitope peptide, was conjugated to the $(I27)_5$ concatamer via the only cysteine residue in the protein (C47 in I27 domain 3, presented schematically in Fig 3A), and the stoichiometry of covalent attachment of the peptide was evaluated by mass spectrometry. A low stoichiometry of peptide attachment to the concatamer (final product mass 54124Da, labelled calibration-38; Fig 3B) was observed on this occasion, which comprises 5.4% of the total preparation. Nevertheless, this low level of conjugation to the concatamer proved sufficient for immunodetection (Fig 3C). Figure 3C shows that antibody SERCA PS-38 recognised the concatamer product decorated with the relevant phosphopeptide (calibration-38), but did not recognise the same concatamer $(I27)_5$ decorated with an irrelevant peptide (calibration-αCLEP) even when 60pmol of concatamer was presented. The calibration standard migrates as a single molecular species of ~60kDa in SDS-PAGE. Furthermore, the phosphorylated epitope was detected by antibody SERCA PS-38 with high

sensitivity, down to a limit of 0.1pmol epitope peptide using standard (SuperSignal West Pico, Pierce) ECL substrate (Figure 3C).

The calibration standard (calibration-38) contained some minor contaminants. A contaminant of 51230Da, seen on the mass spectrum, does not appear to accept peptide (not detected in Western blot experiments, Fig 3C). This material was included in the calculation of percentage product (calibration-38) as it made an appreciable contribution to total protein. A second contaminant of the $(I27)_5$ preparation is covalently labelled by peptide. It underlies the immunostaining of a complex of high Mr (~250kDa, Fig 3C). This product was undetectable in the mass spectrum and therefore is present in low amounts in the calibration-38 preparation. It does not make an appreciable contribution to total protein and was excluded from consideration in the quantification performed in this study.

Thus we conclude that SERCA phosphorylation, if occurring at all, results in the generation of less than 0.03pmol Ser-38 phosphoprotein in the cells studied (10,000 viable myocytes). In previous studies, Ser-16 phosphorylation of phospholamban in myocytes following similar interventions was quantified at 8.5pmol/1,000 cells (7) indicating the presence of at least 85pmol phospholamban in the 10,000 cells of the present study. As phospholamban and SERCA are expressed in similar amounts in cardiac muscle (2 phospholamban per SERCA, (1), we might expect 42pmol of SERCA in the experiments performed. Our failure to detect Ser-38 phosphoprotein with the antibody described herein suggests that less than 0.1% of SERCA is phosphorylated in rat cardiac myocytes treated with CaMKII stimulants.

The present study has described a polyclonal antibody wholly specific for a phosphorylated Ser-38 epitope on SERCA2. The antibody was able to detect the phosphorylated epitope in a calibration standard with high sensitivity (0.03-0.1pmol). However, it failed to recognise SERCA2 in cardiac SR samples from a variety of animal species, despite the presentation of large amounts of SERCA (10-60pmol) and the presence of a phosphoprotein of 100kDa. This indicates that either SERCA is

not phosphorylated on Ser-38, or that only a minor fraction of SERCA molecules (i.e less than ~1%) are phosphorylated on Ser-38. CaMKII activation in isolated cardiac myocytes was achieved using four independent stimuli resulting in phospholamban phosphorylation on Thr-17. None of these resulted in detectable Ser-38 phosphorylation of SERCA using this antibody, despite immunodetection of 0.03pmol of the calibration standard in the same experiment. This indicates that between 0% and 0.1% of SERCA molecules become phosphorylated on Ser-38 in response to CaMKII activating stimuli in intact cardiac myocytes. This study does not provide evidence that Ser-38 phosphorylation of SERCA2a is a significant event in cardiac myocytes or cardiac SR preparations.

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CLAIMS

1. A method of quantifying the amount of target moiety in a sample which may contain the target moiety, the method comprising:

a) providing a presentation system which comprises at least one copy of the target moiety or part thereof that is recognisable by a binding partner and at least one domain which is non-reactive to said binding partner;

b) carrying out a separation based detection technique on said presentation system, wherein said presentation system is present in a specific concentration ;

c) generating at least one comparison point comprising intensity of a signal produced by the presentation system in said technique versus the concentration of the presentation system.

2. A method according to claim 1 wherein the presentation system is present in a single specific concentration.

3. A method according to claim 1 wherein the presentation system is present in a series of varying concentrations.

4. A method according to claim 3, wherein the comparison point is a plurality of comparison points which together provide a calibration curve.

5. A method according to any preceding claim further comprising comparing the comparison point or comparison points with the sample to quantify the amount of target moiety present in the sample.

6. A method according to any preceding claim, wherein said presentation system is of a known molecular weight.

7. A method according to any preceding claim, wherein the presentation system comprises a nucleic acid molecule, a peptide or protein.

8. A method according to any preceding claim, wherein the presentation system comprises a plurality of domains linked in tandem.

9. A method according to any preceding claim, wherein the presentation system comprises identical units or domains.

10. A method according to any of claims 1 to 8, wherein the presentation system comprises non-identical or different units or domains.

11. A method according to any preceding claim wherein the unit(s) of the presentation system are non-reactive to the binding partner specific to the target moiety of part thereof.

12. A method according to any preceding claim wherein the target moiety or part thereof comprises sequences of DNA, RNA or peptide, saccharides, haptens , phosphate, nitrosylated groups, sulphated groups, GPI groups, an epitope or antigenic sequences.

13. A method according to claim 12 wherein the target moiety comprises SERCA2a or SERCA2a phosphorylated on serine-38.

14. A method according to any preceding claim wherein the presentation system comprises differing target moieties or parts thereof.

15. A method according to any preceding claim, wherein the target moiety or part thereof is continuous or discontinuous within the presentation system.

16. A method according to any preceding claim, wherein the specific binding partner comprises a molecule which has a specific binding affinity for the target moiety and is capable of binding thereto.

17. A method according to claim 16 wherein the binding partner comprises an antibody, DNA sequence, RNA sequence or a dye or a drug molecule.

18. A method according to any preceding claim, wherein the separation based detection technique comprises blot based detection techniques, dot blots, Western blot, RIA, fluorescence polarisation, Northern blotting, Southern blotting, PCR, High Performance Liquid Chromatography (HPLC), capilliary electrophoresis, 1D electrophoresis, isoelectric focusing or combinations of the above.

19. A product for use in quantifying the amount of a target moiety which may be present in a sample, the product comprising a presentation system, said presentation system comprising at least one copy of a target moiety or part thereof and further comprising at least one domain linked to said target moiety, wherein the domain(s) is/ are of known molecular weight and are non-reactive to a binding partner specific to said target moiety or part thereof.

20. A kit for quantifying the amount of a target moiety in a sample, the kit comprising a presentation system, said presentation system comprising at least one copy of a target moiety or part thereof and further comprising at least one domain linked to said target moiety, wherein the domain(s) is/ are non-reactive to a binding partner specific to said target moiety or part thereof.

21. A kit according to claim 20 wherein the presentation system is of known molecular weight.

22. The kit according to claim 20 and claim 21 further comprising a binding partner specific to the target moiety or part thereof.

23. The kit according to any of claims 20 to 22, further comprising any one or more of the features disclosed in any of claims 2 to 18.

24. The kit according to any of claims 20 to 23, further comprising instructions for use thereof.

25. The use of a product for quantifying the amount of a target moiety which may be present in a sample, the product comprising a presentation system, said presentation system comprising at least one copy of a target moiety or part thereof and further comprising at least one domain linked to said target moiety, wherein the domain(s) are non-reactive to a binding partner specific to said target moiety or part thereof.

26. The use according to claim 25 wherein the presentation system is of known molecular weight.

27. A method for quantifying an amount of a peptide epitope in a sample, said method comprising:

(a) providing a protein presentation system comprising at least one copy of the peptide epitope and at least one further peptide domain;

b) carrying out a Western blot experiment on said presentation system, wherein said presentation system is in varying concentrations; wherein said Western blot experiment utilises a binding partner specific to the peptide epitope; and further wherein said peptide domain of the presentation system is non-reactive to the binding partner; and

c) generating a calibration curve comprising intensity of a signal produced by the presentation system in said technique versus the concentration of the presentation system.

28. A method according to claim 27, wherein the presentation system is of known molecular weight.

29. A method according to claim 27 or claim 28, wherein the number of target moieties within the presentation system is controlled by selecting peptide domains with a desired number of reactive residues for covalent attachment of the target moiety copy to the domain(s).

30. A method according to claim 29, wherein one site for covalent attachment of the peptide epitope to the peptide domain is provided in the presentation system.

31. A method for quantifying the amount of SERCA2a protein in a sample, the method comprising:

a) providing a protein which comprises at least one copy of an epitope of SERCA2a that is recognisable by an antibody and at least one I27 domain from titin protein which is non-reactive to said antibody;;

b) carrying out a Western blot on said protein, wherein said protein is in a series of varying concentrations;

c) generating a calibration curve comprising intensity of a signal produced by the protein versus the concentration of the protein.

32. A method according to claim 31, wherein the protein is of a known molecular weight.

33. A method according to claim 31 or claim 32, further comprising using the calibration curve to determine the amount of SERCA2a protein present in a sample by comparison of the intensity of signal produced by the sample with the calibration curve.

34. A method according to any of claims 31 to 33, wherein the epitope comprises the amino acid sequence YLEPAILE.

35. A method according to any of claims 31 to 33, wherein the SERCA2a protein is phosphorylated on serine-38 and further wherein the protein comprises an epitope of the phosphorylated SERCA2a protein.

36. A method according to claim 35 wherein the epitope comprises the amino acid sequence ^{31}KLKERWGS(PO$_4$)NEL.^{41}

ABSTRACT

The present invention provides a method of quantifying a target moiety in a sample which may contain the target moiety, the method comprising using a specified concentration or varying the concentration of a presentation system in order to generate a comparison point or calibration curve which provides means for comparing a signal generated by the presentation system and a signal generated by a sample, wherein said presentation system comprises at least one copy of said target moiety or part thereof. Also provided are kits to carry out the method.

Figure 1

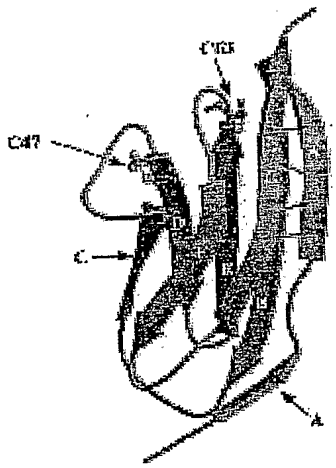
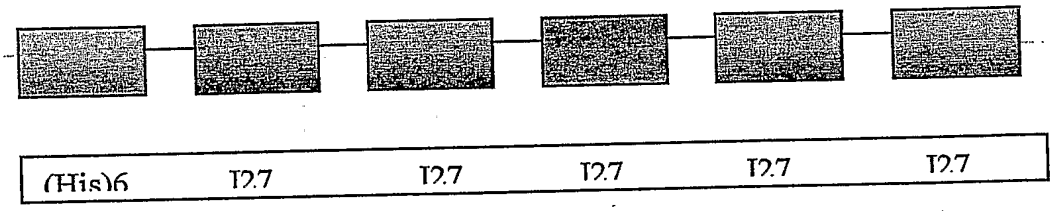
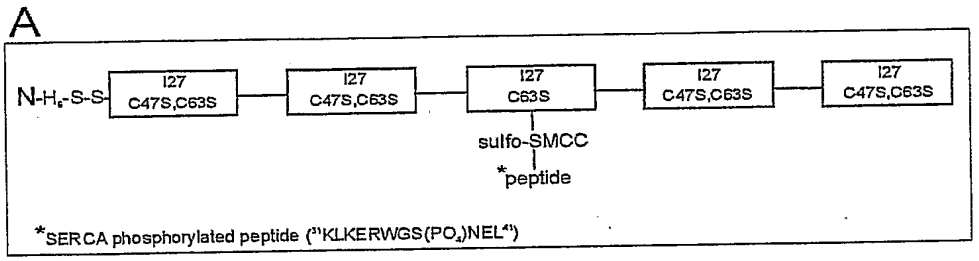


Figure 2

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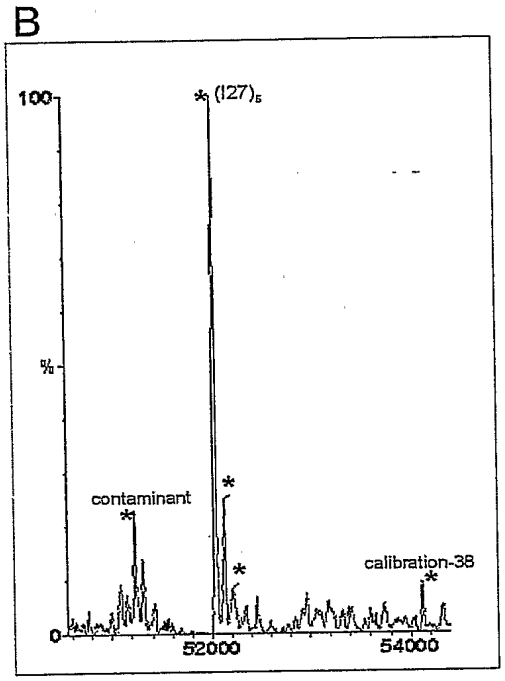
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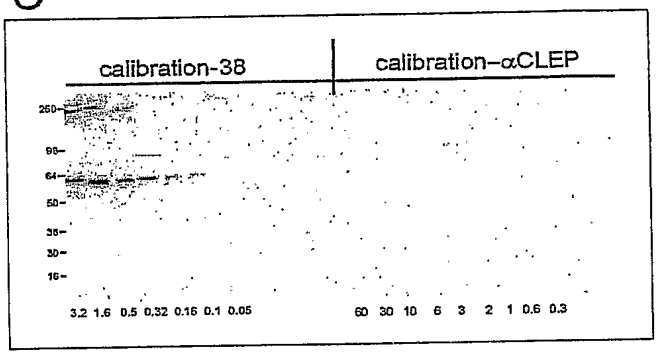
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Figure 3

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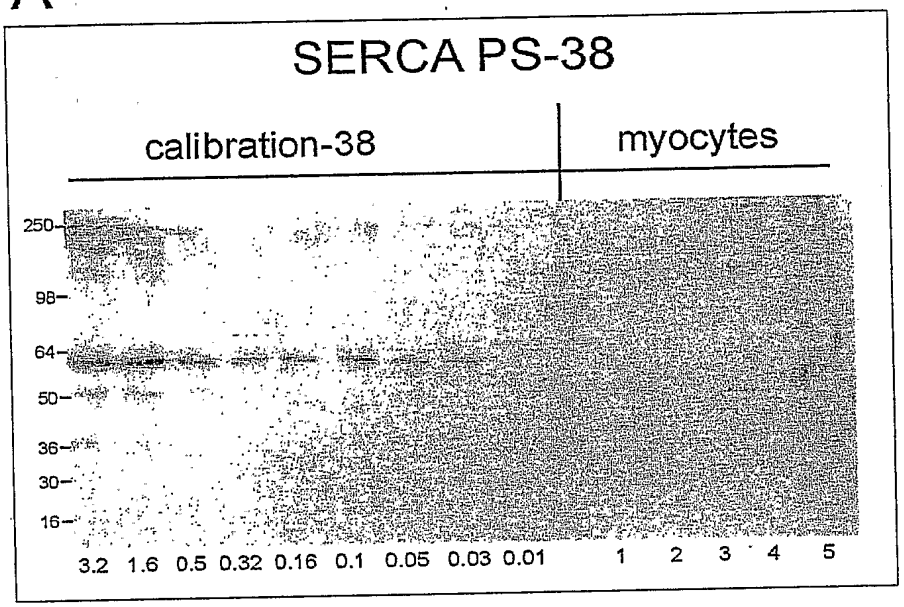
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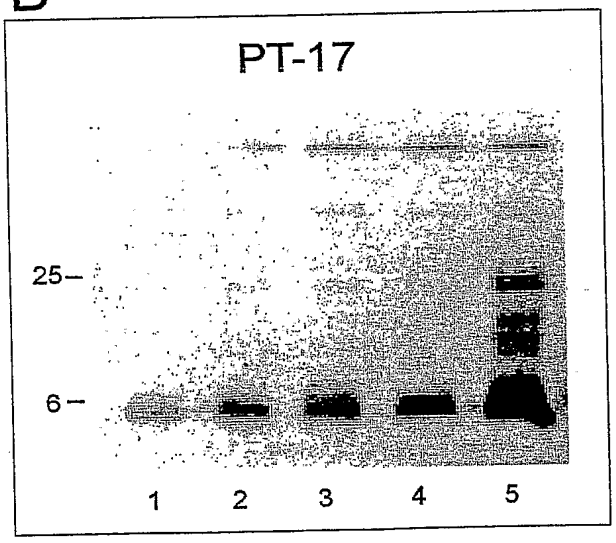
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Figure 4

Figure 5